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Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells

Chang H. Kim [a], Younghoon Oh [a], Tae H. Lee [b,*]

[a] *Biotech Research Institute, LG Chem, Yusung-Gu, Taejeon, South Korea*
[b] *Department of Biology, Yonsei University, Sudaemoon-Gu, Seoul, South Korea*

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Abstract

Codon bias has been observed in many species. The usage of selective codons in a given gene is positively correlated with its expression efficiency. As an experimental approach to study codon-usage effects on heterologous gene expression in mammalian cells, we designed two human erythropoietin (EPO) genes, one in which native codons were systematically substituted with codons frequently found in highly expressed human genes and the other with codons prevalent in yeast genes. Relative performances of the re-engineered EPO genes were evaluated with various combinations of promoters and signal leader sequences. Under the comparable set of combinations, mature EPO gene with human high-frequency codons gave a considerably higher level of expression than that with yeast high-frequency codons. However, the levels of EPO expression varied, depending on the alternate combinations. Since the promoters and the signal leader sequences that we used are known to be equally efficient in gene expression, we hypothesized that the varied expression levels were due to the linear sequence between the promoter and the coding gene sequence. To test this possibility, we designed the EPO gene with hybrid codon usage in which the 5′-proximal region of the EPO gene was synthesized with yeast-biased codons and the rest with human-biased codons. This codon-usage hybrid EPO gene substantially enhanced the level of EPO transcripts and proteins up to 2.9-fold and 13.8-fold, respectively, when compared to the level reached by the original counterpart. Our results suggest that the linear sequence between the promoter and the 5′-proximal region of a gene plays an important role in achieving high-level expression in mammalian cells. © 1997 Elsevier Science B.V.

Keywords: High-frequency codons; Promoter; Signal leader sequence; GC content

1. Introduction

The level of gene expression of eukaryotic genes introduced into mammalian cells depends on various factors such as gene copy number, transcriptional control elements, the site of chromosomal integration, mRNA stability and translational efficiency. Considerable efforts to optimize the level of protein expression in mammalian cells have been concentrated on elements involved in gene copy number and transcription determining elements. However, the study of expression levels of a large number of individual genes has demonstrated that translational events play important roles in limiting the expression of a given gene (Gross and Hauser, 1995). For gene expression, all steps up to transcription are independent of the protein coding sequence and therefore can be adjusted by manipulating the vector construction, gene transfer method and selection protocol. In contrast, control of gene expression at the translational levels is mostly governed by the coding gene structure. However, the mechanisms controlling this type of regulation are often unknown or appear to be complex. Some success in increasing the yield of protein expression under the control of a given promoter has been obtained by introducing an intron sequence that directs the pre-mRNA into the processing/splicing pathway (Petitclerc et al., 1995), by introducing

* Corresponding author. Tel: +82 2 3614084; Fax: +82 2 3125657; e-mail: thlee@bubble.yonsei.ac.kr

Abbreviations: *AdMLp*, adenovirus major late promoter; *CD5L*, native signal leader sequence of CD5 antigen; CHO, Chinese hamster ovary cell; *CMVp*, human cytomegalovirus immediate early promoter; DEAE, diethyl aminoethyl; EPO, human erythropoietin; *EPOL*, native signal leader sequence of EPO; *EPOL^y*, EPO signal leader sequence with yeast codon usage; hGS, human glutamine synthetase; *EPO^h*, *EPO^y*, mature EPO coding sequence with human, yeast prevalent codons; MSX, methionine sulphoximine; PAGE, polyacylamide gel electrophoresis; PCR, polymerase chain reaction; *PolyA*, sequence for polyadenylation; SDS, sodium dodecylsulfate; TK, thymidine kinase; U, unit(s); *UTR*, untranslated region.

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sequences that facilitate translation of mRNA, such as the Kozak consensus sequence (Kozak, 1987), or by manipulating the signal leader peptide of the recombinant protein (Murphy et al., 1993). Another way to increase the protein yield is to modify the coding sequence of an individual gene without altering the amino acid sequence of the gene product (Makoff et al., 1989). This strategy has been used in the past to improve expression of genes from other organisms in *E. coli* (Williams et al., 1988); similar studies have not yet been extensively exercised in mammalian systems.

It is known that the choice of synonymous codons in many species is strongly biased and that a correlation exists between high expression and the use of selective codons in a given organism (Holm, 1986). Efficient expression of the codon-optimized gene can be attributed not only to the abundance of isoacceptor tRNAs and modified nucleotides at the anticodon wobble position available in a host, but also to the formation of a secondary structure of the transcripts favorable for translation. Fig. 1 illustrates that highly expressed human and yeast genes show non-random codon-usage patterns. As noted, the human prevalent codons usually have C or G at their third degenerative position, whereas the yeast-prevalent codons have A or T. Thus, sequence engineering with human codon usage can result in stable mRNA secondary structures because of stronger GC base pairing. However, genes re-engineered with the yeast prevalent codons can form a less stable secondary structure of the transcript. We thought that comparison of performance of the genes re-engineered with either the human or yeast favored codons will provide useful information about the factors affecting gene expression with respect to codon usage and mRNA secondary structure. Here, we constructed various combinations of promoters, signal sequences and synthetic mature EPO genes with human or yeast codon usage, and compared their relative potency in transient expression systems using 293T cells. We showed that the highest expression was obtained with a codon usage-hybrid EPO gene comprising the 5′-segment downstream of the initiator codon with the yeast codon usage and the rest with the human codon usage.

2. Materials and methods

2.1. Generation of EPO synthetic genes

The synthetic, mature EPO genes based on either human or yeast high frequency codons were assembled from eight 80–90 base oligonucleotides that were synthesized by a Applied Biosystem synthesizer (Fig. 2). The eight oligonucleotides contained overlapping 15–20 bases mutually complementary to one another so that they can be utilized for PCR priming. The sequences encoding N-terminal and C-terminal half fragments of

	human	yeast
Ala	GCU	17	**38**
	C	**53**	22
	A	13	29
	G	17	11
Arg	CGU	7	14
	C	**37**	6
	A	6	7
	G	21	4
	AGA	10	**48**
	G	18	21
Asn	AAU	22	**59**
	AAC	**78**	41
Asp	GAU	25	**63**
	C	**75**	37
Cys	UGU	32	**63**
	C	**68**	37
Gln	CAA	12	**69**
	G	**88**	31
Glu	GAA	25	**71**
	G	**75**	29
Gly	GGU	12	**48**
	C	**50**	19
	A	14	21
	G	24	12
His	CAU	21	**63**
	C	**79**	37
Leu	CUU	5	17
	C	26	5
	A	3	13
	G	**58**	10
	UUA	2	**28**
	G	6	28
Lys	AAA	18	**58**
	G	**82**	42
Pro	CCU	19	31
	C	**48**	15
	A	16	**42**
	G	17	12
Phe	UUU	20	**60**
	C	**80**	40
Ser	UCU	13	**26**
	C	28	16
	A	5	21
	G	9	10
	AGU	10	16
	C	**34**	11
Thr	ACU	14	**35**
	C	**57**	20
	A	14	31
	G	15	14
Tyr	UAU	26	**56**
	C	**74**	44
Val	GUU	7	**39**
	C	25	21
	A	5	21
	G	**64**	19

Fig. 1. Codon usage of highly expressed human and yeast genes. Percentage frequencies of the synonymous codons are shown for each corresponding amino acid. The most prevalent codon is shown in bold.

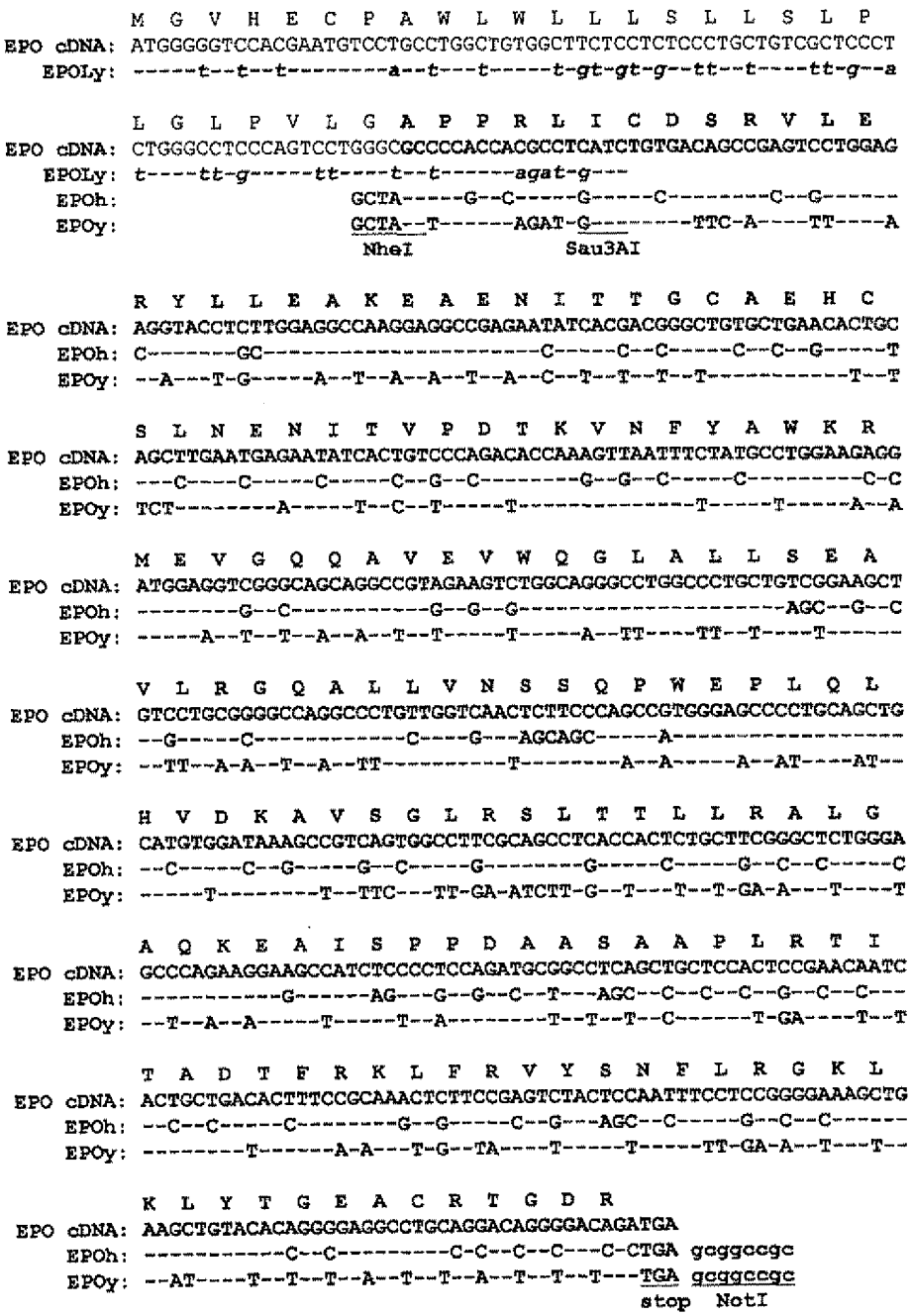

Fig. 2. Nucleotide sequence of the EPO cDNA and the mature EPO genes with human and yeast codon usage (*EPO*^{h} and *EPO*^{y}). The deduced amino acid sequence shown above each codon is designated by the single letter code. Nucleotide and amino acid sequences of mature EPO are shown in bold. The substituted nucleotides of the synthetic mature EPO genes (*EPO*^{h} and *EPO*^{y}) are shown below the EPO cDNA sequence in two lines. The italicized nucleotides indicate the yeast codon-based synthetic sequence encoding the EPO leader peptide and consecutive six amino acids (*EPOL*^{y}). The sites of the restriction enzymes used for cloning are also indicated above.

EPO gene were separately generated by the first PCR. Typically, PCR was conducted using 30 cycles with an annealing temperature of 50°C and 30-s extension time. Small aliquots of the two PCR reactions were mixed and subjected to PCR for five rounds in the same condition described above, and then primers, each of which contained a sequence for a restriction site (*Nhe*I or *Not*I) and an adjacent sequence complementary to the 5′ or 3′ end of the mature EPO gene, were subsequently added and amplified further by PCR for 30 cycles to generate the full-sized mature EPO genes (Fig. 3).

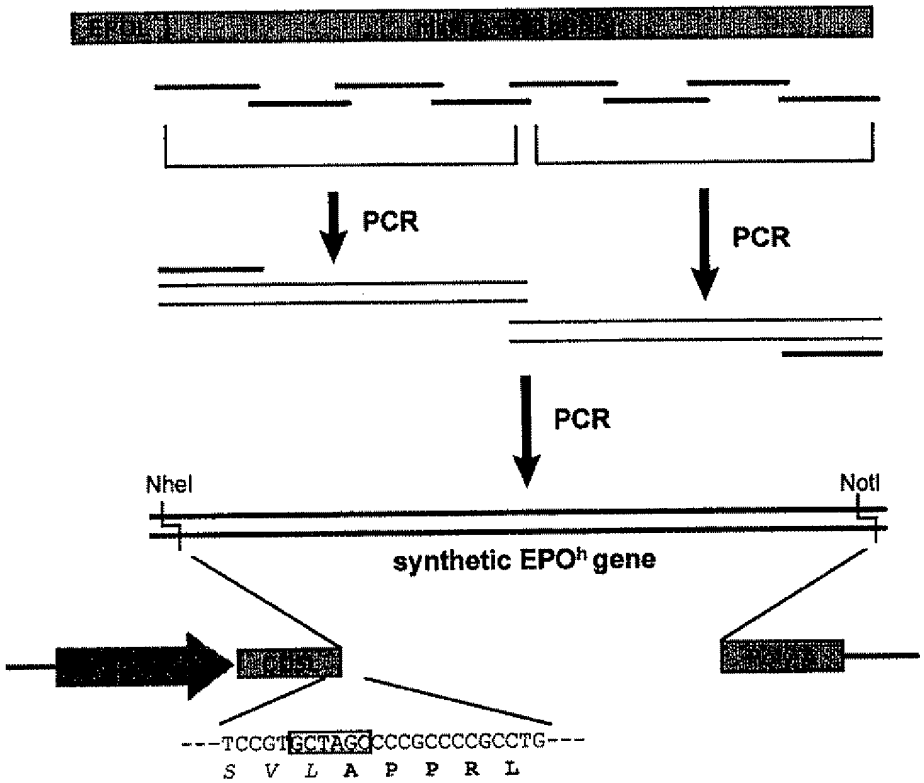

Fig. 3. Schematic diagram of PCR synthesis and cloning strategy of a mature EPO gene with human or yeast codon usage. The unique restriction sites used for cloning are shown. The shaded boxes in the expression construct driven by the CMV promoter denote the leader peptide (CD5L) and polyadenylation site (Poly A). The nucleotide and encoded amino acid sequence at the junction between CD5 signal leader sequence (italicized) and the mature synthetic EPO gene (bold) are shown. The box in the nucleotide sequence indicates a unique *Nhe*I restriction enzyme site.

2.2. Plasmid constructions

The PCR products were gel-purified, phenol-extracted, and excised with restriction enzymes, *Nhe*I and *Not*I. The fragments were cloned into a CDM7-derived plasmid containing a leader sequence of the CD5 surface antigen (Aruffo et al., 1990), resulting in pCDM–CD5L–EPO^h or –EPO^y. The correct sequences were confirmed by DNA sequencing. To generate adenovirus major late (AdML) promoter-driven plasmids, AdML promoter retaining 180 bp of the first two and two-thirds of the third leaders of adenovirus major late mRNAs was PCR-amplified from an expression vector, a pED derivative (Kaufman et al., 1991), and was subsequently replaced with the human cytomegalovirus (CMV) promoter in thepCDM–CD5L–EPO^h construct, generating pAdML–CD5L–EPO^h. To construct plasmids containing a natural leader sequence of EPO (*EPOL*), two complementary oligonucleotides were synthesized, based on the sequence shown in Fig. 2, and annealed. The annealed fragment was inserted into the *Xho*I/*Nhe*I cut EPO expression plasmids described above, resulting in pCDM–EPOL–EPO^h (or –EPO^y) and pAdML–EPOL–EPO^h (or –EPO^y). We also synthesized two complementary oligonucleotides encoding the EPOL and six additional amino acids of mature EPO based on the yeast prevalent codons (denoted in Fig. 2 as *EPOL^y*). The annealed fragment, which has *Xho*I and *Sau*3AI compatible sites at its 5′ and 3′-ends, respectively, was subsequently inserted into the *Xho*I/*Not*I-cut plasmids carrying either CMV or AdML promoter, along with the *Sau*3AI/*Not*I fragment of *EPO^h*, resulting in pCDM–EPOL^y–EPO^h and pAdML–EPOL^y–EPO^h. To generate a plasmid for stable expression, pCDM–EPOL^y–EPO^h was manipulated to contain the thymidine kinase (*TK*) promoter and human glutamine synthetase (*hGS*) at its *Bgl*II and *Sal*I sites. The 1.1 kb of hGS sequence was PCR-amplified from the human liver cDNA library based on the known sequence (Gibbs et al., 1987).

2.3. Transient transfections and selection of EPO-producing stable CHO-K1 lines

The expression constructs were transiently transfected into 293T cells by the DEAE-dextran method as described elsewhere. For stable EPO expression, the calcium phosphate precipitation method was used to transfect the pCDM–EPOL^y–EPO^h containing an hGS cDNA as a selection marker, into the CHO-K1. Two

rounds of gene amplification with methionine sulphoximine (MSX) (Sigma) were carried out to select for EPO-expressing CHO-K1 cell lines. The detailed selection method was described by Cockett et al. (1990).

2.4. Measurement of EPO

The expression level of supernatants from 293T cells transiently transfected with each EPO expression vectors was assessed by Western analysis, and biological activity was measured by in vitro cell proliferation. For Western blot analysis, the culture supernatants were harvested 72 h post-transfection, and the equal volumes (20 μl) were fractionated on 12% SDS–PAGE and transferred to PVDF membrane using a Bio-Rad apparatus. Immunoblotting was carried out using a rabbit polyclonal antiserum raised against recombinant human EPO (Genzyme) as described elsewhere. Biological activity of the culture supernatants was determined using spleen cells from mice rendered anemic by treatment with phenylhydrazine hydrochloride. The detailed procedure was described by Krystal (1983). A recombinant EPO from Boeringer-Manheim was used as a reference to determine relative EPO units of the culture supernatants.

3. Results and discussion

3.1. Synthesis of EPO genes based on human or yeast codon usage

Human or yeast-prevalent codons shown in bold percentage frequencies (Fig. 1) were mainly chosen to generate two synthetic mature EPO genes (*EPO*^{h} and *EPO*^{y}) illustrated in Fig. 2. Some deviations from strict adherence to prevalent codon usage were made to accommodate the introduction of unique restriction sites or to avoid homopolymeric DNA sequences. Each synthetic EPO gene was assembled from mutually priming long oligonucleotides that were subsequently amplified by two-stage PCR, as schematically depicted in Fig. 3. The resulting two synthetic leaderless EPO genes were inserted downstream of the sequence encoding the leader peptide of the human CD5 antigen (*CD5L*) in the expression vector (Aruffo et al., 1990), where the CMV promoter directs the transcription of the chimeric EPO precursor genes.

3.2. Expression of the human or yeast prevalent codon-based EPO genes

To evaluate the relative potency of the human and yeast prevalent codon-based EPO genes, we compared the results of transient transfection of the two EPO expression constructs. The EPO expression level of the supernatants was assessed either by Western blot analysis using a rabbit polyclonal anti-EPO antibody or in-vitro cell proliferation assay. As shown in Fig. 4, the expression plasmid containing the EPO gene with the human high frequency codon (*EPO*^{h}) directed the synthesis of EPO more efficiently than the plasmid with yeast prevalent codon-based EPO gene (*EPO*^{y}), shown by their expression levels, which were 37.2 and 14.7 U/ml, respectively. The expressed EPO was tested to be biologically active, and it migrated with the molecular weight of 34 kDa on SDS–PAGE, which represents the glycosylated form (Dube et al., 1988).

3.3. Comparative study using factors that affect the expression of EPO gene expression

Various combinations of promoters, signal sequences and synthetic EPO genes with different codon usage were comparatively tested for their ability to drive the synthesis and secretion of EPO in 293T cells. We chose the chimeric *CD5L–EPO*^{h} as the reference EPO gene for comparison. Although it is known that both the CMV promoter and adenovirus major late (AdML) promoter are equally strong in heterologous gene expression, we initially tested which promoter would be more advantageous in the expression of the CD5L–EPO^h gene. To do this, we cloned the AdML promoter, which includes the tripartite leader sequence, and subsequently replaced it with the CMV promoter of pCDM–CD5L–EPO^h. As shown in Fig. 5A, the AdML promoter directed the CD5–EPO^h gene expression (34 U/ml) equivalent to that of the CMV promoter (37 U/ml), indicating that there was no difference in the potency of two promoters to drive the CD5L–EPO^h gene expression. Next, we examined the effect of the signal leader sequences on the EPO^h gene expression. We replaced the CD5L sequence with the natural EPO leader sequence (*EPOL*). A representative transfection result, as shown in Fig. 5B, revealed that under the CMV promoter, the natural *EPOL* drove the EPO^h gene expression slightly better than the *CD5L*. We also constructed another combination in which the natural *EPOL* was joined to the mature EPO gene with the yeast codon usage (*EPOL–EPO*^{y}). As shown in Fig. 5C, under the CMV promoter, the expression of EPOL–EPO^y gene was increased by 2.6-fold (97 U/ml), compared to the reference CD5L–EPO^h gene (37 U/ml). To our surprise, under the AdML promoter, the expression of EPOL–EPO^y gene was further enhanced up to 290 U/ml, which represents a 7.8-fold increase in comparison with the reference construct (*CMVp–CD5L–EPO*^{h}). Since we have shown that both the CMV promoter and the AdML promoter have a similar strength to drive the expression of CD5L–EPO^h gene (Fig. 5A) and the EPO^h gene performed better than the EPO^y gene under the CMV promoter (Fig. 4), neither the promoter strength nor the effectiveness of coding

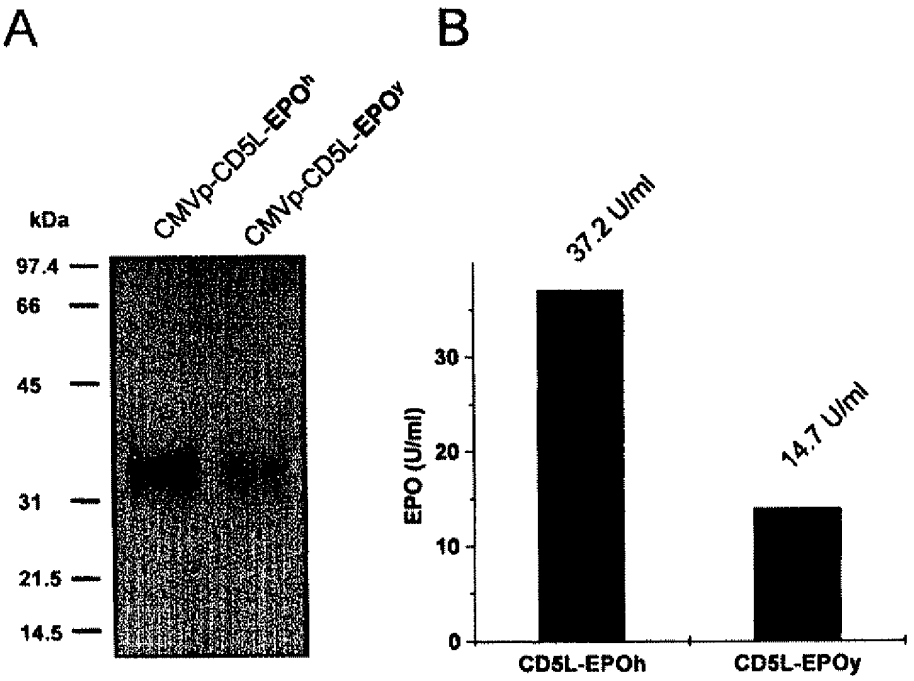

Fig. 4. Expression of the synthetic EPO genes in transient transfection assay in 293T cells. (A) Western analysis of supernatants from 293T cells transfected with plasmids containing expression cassettes arrayed in CMV promoter–CD5L–synthetic EPO gene with human codons (*CMVp–CD5L–EPO^h*) or with yeast codons (*CMVp–CD5L–EPO^y*). Representative results of three independent experiments are shown. (B) In vitro cell proliferation assay of supernatants from transiently transfected cells. The same culture supernatants were tested for their ability to stimulate ^{3}H-thymidine uptake using spleen cell from mice rendered anemic by treatment with phenylhydrazine. The relative EPO bioactivity (U/ml) was deduced from the total incorporated radioactivity by using a reference EPO. The results shown are averages of triplicate experiments, which differed by less than 10%.

gene sequence could account for the substantial increase of the EPOL–EPO^y gene expression by the AdML promoter. In addition, it has been reported that the CD5 signal leader peptide efficiently directs the synthesis and the export of secreted and membrane-bound proteins (Aruffo et al., 1990). Thus, a slightly improved performance of the EPO leader to facilitate the expression of EPO^h gene could also hardly account for the substantially increased expression of the EPO^y gene. Therefore, the variation of EPO expression levels dependent on the combinations of promoter, signal leader sequence or synthetic EPO gene could be explained by the notion that the contextual linear sequence between the promoter, and the adjacent 5′-terminal coding region of EPO gene may be an important factor for gene expression.

3.4. Enhancement of EPO expression using an EPO gene with yeast–human hybrid codon usage

So far, we have obtained the highest expression using the EPOL–EPO^y gene under the control of the AdML promoter. Granting that our results show an equal strength of CMV promoter and AdML promoter as well as a better performance of mature *EPO^h* over *EPO^y* in separate experimental sets, there must be room for improvement of EPO expression by using the CMV promoter and mature EPO^h gene, at least comparable to the level reached by using the AdML promoter and EPO^y gene. Although several factors involving in the control of gene copy number, transcription and translation are attributed to the overall expression efficiency, it is known that mRNA with a high GC content of the 5′-untranslated region (*UTR*) may be translated with low efficiency (Southard et al., 1995). We hypothesized that the high GC content of the region downstream of the initiator codon, not to mention in the 5′-*UTR*, also may impair translation efficiency. As noted in Section 1, human prevalent codons always have C or G at their degenerative third-bases, whereas yeast prevalent codons adopt A or T. Therefore, a given gene optimized with human codon usage becomes high in GC content. This high degree of GC content, particularly in the promoter proximal region may be disadvantageous in gene expression in mammalian cells. We, therefore, predicted that decreasing the GC content of the limited region downstream of the initiator codon of the EPOL–EPO^h gene could result in an increased EPO expression. To test this possibility, we made another EPO gene with a hybrid codon usage in which the 5′-proximal region of the EPO gene containing yeast high-frequency codons (EPO leader sequence plus the sequence encoding consecutive

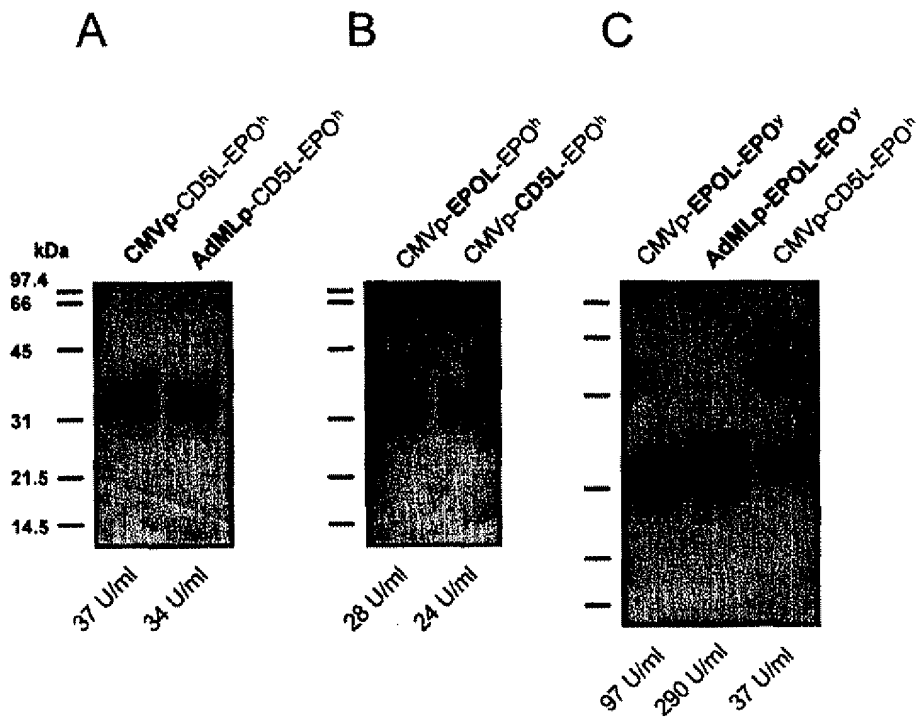

Fig. 5. Expression of EPO in various combinations of promoters, signal leader sequences and synthetic EPO genes. The promoter-signal leader-synthetic EPO gene combination of each plasmid is indicated. The bioactivity of EPO is indicated under each corresponding lane of western blots. (A) Comparison of promoter efficiencies between the CMV promoter (*CMVp*) and adenovirus major late promoter (*AdMLp*). (B) Comparison of CD5L and natural EPOL sequences. (C) Comparison of EPO expression with EPO^y and EPO^h in alternate combinations of promoters and leader sequences. Representative data from at least two experiments are shown. The values of EPO bioassy were the averages of two or three independent experiments, whose standard deviation was less than 10%.

six amino acids, as denoted $EPOL^y$ in Fig. 2) was linked to the rest of the EPO gene with human high-frequency codons. The resulting codon-usage hybrid gene, $EPOL^y$–EPO^h was tested for its performance under the CMV promoter or AdML promoter. Our prediction was essentially borne out, as shown in Fig. 6A. Re-engineered $EPOL^y$–EPO^h gene gave a substantially enhanced EPO expression up to 593 U/ml with the CMV promoter and 540 U/ml with the AdML promoter, which represents a 13.8-fold enhancement compared to the level attainable with *CMVp–EPOL–*EPO^h and a twofold enhancement compared to the level with *AdMLp–EPOL–*EPO^y, respectively. A representative CHO-K1 cell that was permanently transfected with the codon usage hybrid $EPOL^y$–EPO^h could produce biologically active EPO at 10 085 U/ml after two rounds of amplification using human glutamine synthetase as a selection marker (Fig. 6B). RNA slot blot analysis showed that cells tranfected with *CMVp–*$EPOL^y$–EPO^h produced a 2.9-fold higher increase in the level of the transcripts than those with *CMVp–EPOL–*EPO^h, judging by the laser densitometry scanning of the autoradiogram shown in Fig. 7. In view of the 13.8-fold increase in EPO yield by *CMVp–*$EPOL^y$–EPO^h compared to *CMVp–EPOL–*EPO^h, these data suggest that the enhanced efficiency of expression could be attributable to the multiple factors such as enhanced transcription, translational efficiency, or increased mRNA stability.

From the outcomes of replacement experiments of the promoters, signal leaders and synthetic EPO coding sequences, as well as the successful enhancement of EPO expression by decreasing the GC content of the promoter-proximal coding region, we could tentatively draw empirical guidelines for heterologous gene expression in mammalian cells. Codon usage affects the general expression level of a heterologous gene. Re-engineering the coding sequence to match to the codons frequently found in human genes is beneficial to achieve high-level expression. Recent reports clearly support this. Altering the coding sequence of the HIV envelope glycoprotein gp120 and jellyfish green fluorescent protein genes to the human prevalent codons results in a substantial increase in expression efficiency (Haas et al., 1996; Zolotukhin et al., 1996). Re-engineered genes with human codon usage become high in their GC content. Although a low GC content of *5′-UTR* is ensured, optimizing the re-engineered gene further by decreasing the GC content of the limited region downstream of the initiator codon is advisable.

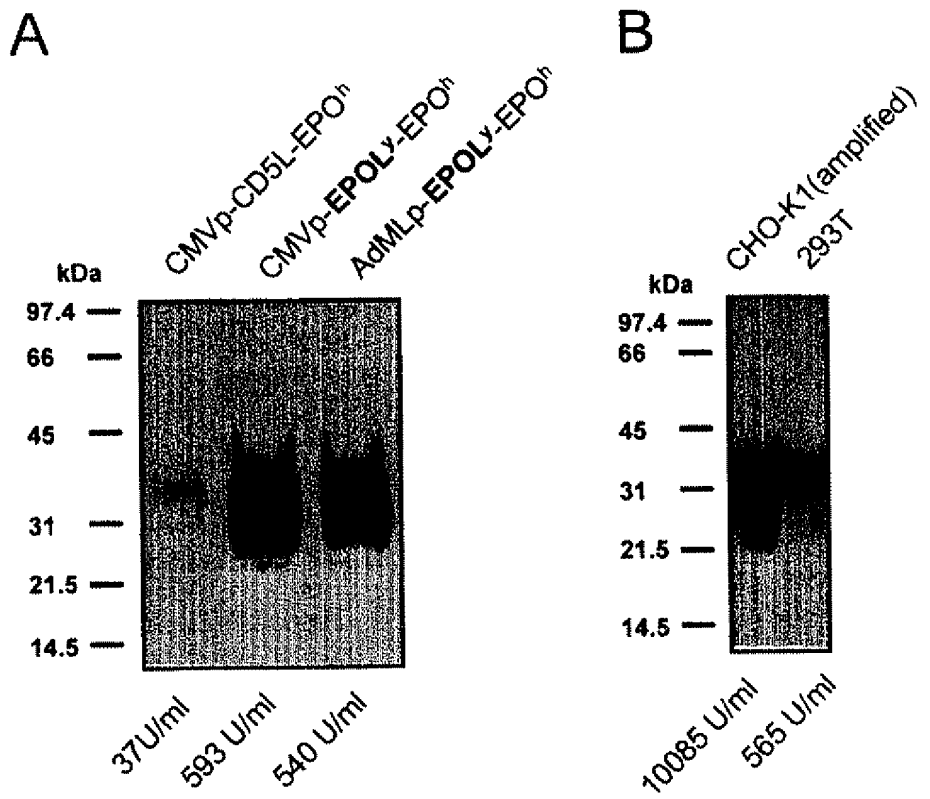

Fig. 6. Enhancement of EPO expression using the codon-optimized EPO gene. (A) Western analysis of supernatants from 293T cells transiently transfected with plasmids containing the codon usage-hybrid EPO gene (*EPOL^y–EPO^h*). (B) Western analysis of supernatant from CHO-K1 cells permanently transfected with the plasmids carrying a codon-usage hybrid EPO gene driven by the CMV promoter (*CMVp–EPOL^y–EPO^h*) as well as the human glutamine synthetase under the TK promoter. The supernatant was harvested from the 3-day-old culture of a CHO-K1 cell line that was selected by two rounds of amplification, and was subjected to Western analysis along with the supernatant from the culture of 293T cells transiently transfected with the same plasmids. The standard deviation observed between triplicate EPO measures was 5% or less.

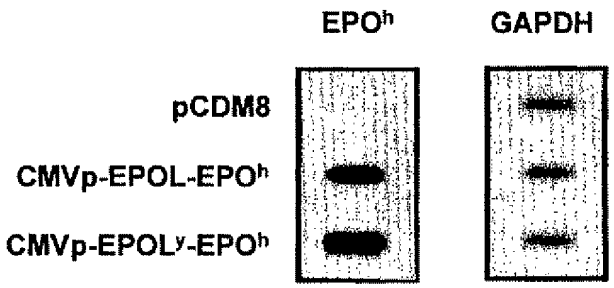

Fig. 7. RNA slot blot analysis of 293T cells transfected with the control plasmid (pCDM8), pCMV–EPOL–EPO^h, or pCMV–EPOL^y–EPO^h. Five micrograms of cytoplasmic RNA samples prepared from each transfectant were blotted and hybridized with ^{32}P-labeled *EPO^h* probe and with ^{32}P-labeled *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) probe as a control. Images of autoradiogram are shown.

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